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Morphological and Molecular Characterization of Stunt Nematodes of Agricultural Importance

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Abstract

Stunt nematodes belong to an important group that feeds on the roots of many types of crops worldwide. They include *Tylenchorhynchus* spp. and *Merlinius* spp., which although, at present are classified as minor pests, but are a potential biosecurity threats to global agriculture production and food security. Molecular and morphological studies of *Tylenchorhynchus thermophilus, T. annulatus* and *Merlinius brevidens* were conducted in this study with the main aim of accurate identification and classification of the pest. This is necessary in planning of protective and preventive measures to reduce the risk of transmission of these nematodes. The phylogenetic relationships were analyzed using ITS 1 and 18S genes of ribosomal DNA. These analyses indicated that sequence identity ranged from 97.1% to 99.8% among six *Merlinius* populations and 91.4% to 98.9% between two *Tylenchorhynchus* populations. The results of this study are significant in biosecurity measures at plant quarantine stations at sea and airports and also useful to scientists, extension agencies and farmers involved in nematode research and control. © 2014 Friends Science Publishers

Keywords: Biosecurity; Biotechnology; Molecular nematology; *Tylenchorhynchus*

Introduction

Stunt nematodes feed on the roots of many kinds of plants some of which are of agricultural importance worldwide (Ramzan *et al*., 2008). These nematodes belong to a group consisting of the *Tylenchorhynchus* spp., *Merlinius* spp. and *Amplimerlinius* spp. (Handoo *et al*., 2007; Ramzan *et al*., 2008). Their activity cause moderate to low yield losses in variety of crops such as rice (*Oryza sativa* L.) potato (*Solanum tuberosum*), wheat (*Triticum* spp.), bean (*Vigna* spp.), (Golden *et al*., 1987; Handoo *et al*., 2007; Ramzan *et al*., 2008). These nematodes were originally reported from the US and later found on other parts of the world. For example, *T. quaidi, T. tritici, T. qasimi* and *Merlinus khuzdarensis* were first detected from Pakistan (Golden *et al*., 1987; Handoo *et al*., 2007; Ramzan *et al*., 2008). More than sixteen species of *Tylenchorynchus* spp., some of those are of agricultural importance have been described in Pakistan (Maqbool, 1997). Many species of the genus *Tylenchorhynchus* were described from different parts of the world including Africa (Seinhorst 1963; Venditti and Noel, 1995), Asia (Chen *et al.* 2012; Duan *et al*., 2012), Europe (Hallman *et al.*, 2007) and Australasia (Hodda and Nobbs, 2008). Effective biosecurity measures involving preventive control of these nematode species through quarantine and other legislative methods and also protective methods using alternative sustainable control tactics for instance the use of plant materials (Hassan *et al*., 2013) can only be achieved if the species are accurately identified, classified and documented in literature (Barker and Koenning, 1998; Martin *et al*., 2000). Therefore, the main aim of this research is to identify and determine their phylogenetic status based on molecular and morphometric characteristics.

Materials and Methods

Rhizosphere soils were collected around the indigenous plant roots from three cities in China namely; Beijing, Hangzhou (30.18’052 N, 120.44’901) and Shanxi (37.50’802 N, 111.27’552 E). Plastic bags containing soil samples were taken to the nematology laboratory at Zhejiang University, Hangzhou, China. Nematodes were extracted from 100 g soil samples by decanting and sieving techniques and killed by gentle heat and fixed with a formaldelhyde: glycerin (4:1) solution. They were then examined under Stereo microscope to identify the target species. Morphological measurements were conducted using Lucica program. Permanent slides of these species were deposited at the nematology laboratory at Zhejiang University, China.

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PCR Amplification, Sequence Alignment and Phylogenetic Analyses

Individual nematodes transferred into Eppendorf tubes containing 16 µL ddH_2O. Two µL PCR buffer solution (without $MgCl_2$) were added in the Eppendorf tubes. Nematodes were crushed using a pipette tip, and centrifuged at 12,000 rpm for 1 min, and immediately frozen at minus 68°C for at least 30 min. Tubes were then heated to 85°C for 2 min. and added to 2 µL proteinase K, then incubated at 56°C for 30 min, followed by 10 min at 95°C. After incubation, these tubes were cooled at 4°C for 10 min then stored at minus 21°C until used (Zheng *et al.*, 2003).

A twenty-five microlitres PCR mixture was developed, which contained 2.5 µL LA buffer, 4 µL dNTP, 0.5 µL each primers (synthesized by Takara Company, Shanghai, China) and 1.25 µL DNA template, 0.25 µL LATaq and 16 µL distilled water. All PCR reactions were conducted in an S1000 thermal cycler. Primers used for amplification of ITS1 region were forward primer 5.8S (5'ACGAGCCGAGTGATCCACCG3') and reverse primer 18S (5' TTGATTACGTCCCTGCCCTTT3'). The thermal cycling protocol was as follows: denaturations at 94°C for 4 minutes, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 1 min. A final extension was at 72°C for 10 min. Primers for amplification of 18S region were forward primer 988F (5' CTCAAAGATTAAGCCATGC3') and reverse primer 1912R (5'TTTACGGTCAGAACTAGGG3'). The thermal cycling protocol consisted of denaturations at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 40 s, annealing at 50°C for 45 s, and extension at 68°C for 1.5 min. A final extension was at 68°C for 10 min. After DNA amplification, 3 µL aliquots of the PCR products were resolved by electrophoresis in 1% agarose gel (100V, 400 mA, 30 min), stained in ethidium bromide for 10 min and vizualized on UV illimuniation.

Amplified PCR products were purified as instructed by the nucleic acid purification kit from AXYGEN Company (Hangzhou, China, catalogue No. AP-GX-250). Purified PCR products were ligated to pUCM-T vector and transformed in to DH 5alpha competent cells. PCR amplification was further confirmed with the primer insertion and expected band. Sequencing was done at the SANGON Biotechnology Co., Ltd, Shanghai, China. Alignment of sequences with closely related published sequences, which have query coverage more than 99% and E-value = 0 were retrieved from GenBank database was conducted (Razaq *et al.*, 2013). Sequences were analyzed and aligned using the Clustal W program of the Mega 5.0 and DNA star software. Phylogenetic trees were created by the neighbor joining method and bootstrapping analysis. The sequences were deposited in Genebank with accession numbers and listed in Table 3.

Results

Measurements for all species were shown in Table 1 and 2.

***Tylenchorhynchus thermophilus* (Fig. 1):** Females body shape had ventral curvature after gentle heating. Head rounded, continuous, 5.8 -8.2 µm diam., 2.3 -4 µm height, with three annules. Stylet 19.6 -22 µm long, with rounded stylet knobs 3.7 -5.7 µm diam, 1.5 -2.9 µm height. Dorsal gland orifice (DGO) 1.9 -2.9 µm from stylet base. Excretory pore located at 102.7 -124.3 µm from head end. Four incisures in the lateral field. The vulva was transverse lit and ovaries outstretched. Tail 46.4 -55.9 µm long, with 21 -27 annules. Phasmids prominent, at 30.2 -36.9 µm from tail tip. Males of this specie were not found.

***T. annulatus* (Fig 2):** Females body ventrally arcuate after gentle heating. Head rounded, continuous, 6.8 -8.3 µm in diam. and 2.1- 4 µm height, three distinct annules. Stylet 18.7 -21.4 µm long, with stylet knobs 3.3 -5 µm diam., 1.9 - 2.3 µm height. Excretory pore was located at 99.6 -121.8 µm from head end. Four incisures in the lateral field. Ovaries were outstretched. Tail subcylindrical, 32.4 - 44.5 µm long, with 14 - 20 annules.

Males of this specie were not found *T. annulatus* was found in all continents, except Europe and widely distributed in the US. The morphology and morphometrics of Chinese populations fit the previous descriptions. This is the first record of *T. annulatus* from *Pinus hwangshanensis* in China.

***Merlinius brevidens* (Figs. 3 and 4):** Females: Body shape strongly arcuate after gentle heat. Head rounded, 7- 10 µm diam., 2.8 -5.2 µm height. Strong stylet, 26 -34 µm long with rounded stylet knobs, 4.6 -7.8 µm diam., 2 - 4.3 µm heigh. Excretory pore located at 131 – 151 µm from head end. Six incisures occurred in lateral field. Tail curved, smooth, 40 -76 µm long, containing 46 -61 fine annules. Phasmids near mid tail.

Mophometrics of females of *M. brevidens* from six populations were similar to each other.

Males: All characteristics in the anterior region were similar to females. Each had a distinctive spicule 23.31 ± 2.88 µm (20.87 -26.49 µm), and small gubernaculum.

M. brevidens was originally described from grass in the US. The morphology and morphometrics of Chinese populations did not differ from the original description except longer stylets. This is the first record of *Merlinius* from six indigenous plants in China (Table 2).

Molecular Characterization

Sequence identity ranged from 91.4% to 98.9% between two *Tylenchorhynchus* populations by using ITS1 and 18S regions, respectively. Sequence identity ranged from 97.1% to 99.8% among six *Merlinius* populations by using 18S regions (Fig. 5).

Table 1: Morphometrics of *Tylenchorhynchus thermophilus* Golden, Baldwin and Mundo- Ocampo, 1995 and *T. annulatus* Golden, 1971 from China. Measurement in μm and in form mean ± s.d (range)

Species	*Tylenchorhynchus thermophilus*	*T. annulatus*
N	9	9
L	716.12 ±26.8 (683.89-771.94)	687.42±35.68 (653.93-751.5)
a	30.34±1.42 (27.51-31.6)	27.8±3.27 (21.74-32.26)
b	4.9 ±0.18 (4.65-5.19)	4.89 ±0.17 (4.65-5.17)
c	13.83 ±0.66 (12.94 -14.82)	17.99 ±2.68 (13.55 – 21.26)
c’	3.42 ± 0.39 (2.71 – 4.16)	2.85 ± 0.36 (2.28 – 3.24)
V	55.59±1.59 (54.41-59.66)	55.93 ±1.77 (51.76 – 57.95)
Body diam. at vulva	23.02 ±1.73 (20.38 – 25.45)	24.57±4.04 (20.25 – 33.73)
Stylet length	20.75 ±0.83 (19.56-21.97)	19.89 ±0.8 (18.76 – 21.42)
Stylet knob height	2.18 ±0.49 (1.5-2.9)	2.09 ±0.14 (1.93 – 2.3)
Stylet knob width	4.58 ±0.65 (3.7-5.66)	3.87 ± 0.48 (3.34 – 5.00)
Dorsal Gland Opening	2.45 ±0.35 (1.92 – 2.93)	1.68 ±0.22 (1.51 -2.00)
Head to vulva	398.33 ±24.72 (376.49 – 460.53)	384.45 ±28.05 (338.45 – 427.69)
Body diam. at stylet knob	14.43 ±0.73 (13.66-16.18)	14.48 ±1.28 (13.16 – 17.17)
Body diam. at middle median bulb	19.85 ±0.68 (18.56 – 20.62)	19.93 ±2.33(17.19-24.5)
Distance anterior end – extetory pore	110.56 ±6.85 (102.71 – 124.33)	109.45±8.02 (99.64-121.78)
Tail length	51.92 ±3.54 (46.39- 55.91)	38.97±5.96 (32.4-48.55)

Table 2: Morphometric data of *Merlinius brevidens* from China. Measurement in μm and in form mean ± s.d (range)

Populations	9	10	11	12	13	5
N	10	10	13	9	12	6
L	833.04±53.39 (751.66-913.4)	826.24±56.52 (748.11-924.76)	862.68±72.51 (756.32-997.31)	896.94±69.42 (819.45-995.55)	846.48±98.85 (648.1-947.73)	876.05±68.18 (796.05-984.71)
a	25.29±4.02 (21.81-34.44)	27.81±2.53 (23.74-31.44)	26.32±2.59 (22.44-30.21)	25.51±2.42 (22.47-30.44)	24.85±4.76 (16.22-31.53)	28.2±1.7 (25.42-30.59)
b	5.56±0.27 (5.26-6.02)	5.15±0.28 (4.77-5.64)	5.34±0.4 (4.53-6.00)	5.52±0.21 (5.29-5.84)	5.23±0.36 (4.53-5.78)	5.57±0.47 (5.03-6.11)
c	12.91±0.85 (11.61-14.46)	12.4±1.06 (11.1-14.49)	15.07±3.47 (10.26-20.5)	17.7±2.62 (11.78-20.56)	15.92±2.46 (11.77-20.06)	12.39±0.7 (11.58-13.32)
c’	3.47±0.44 (2.48-4.18)	3.82±0.31 (3.53-4.55)	3.36±0.7 (2.37-4.57)	2.92±0.57 (1.57-3.75)	3.14±0.45 (2.38-4.00)	3.79±0.42 (3.16-4.21)
V	52.89±1.84 (49.21-55.31)	54.56±1.57 (52.6-56.65)	55.63±3.78 (51.63-63.87)	54.23±2.34 (52.36-60.07)	56.94±4.45 (50.35-62.79)	53.74±0.69 (52.72-54.64)
Body diam. at vulva	33.11±4.45 (24.25-38.36)	29.38±3.22 (24.09-34.15)	32.54±3.37 (27.1-37.23)	35.03±2.17 (31.71-37.57)	34.25±4.2 (26.74-40.16)	30.92±3.92 (27.1-38.4)
Stylet length	27.54±1.06 (26.13-29.13)	27.62±1.14 (26.03-29.48)	28.05±1.08 (26.4-30.43)	29.4±1.27 (27.2-31.24)	29.51±0.91 (27.96-30.61)	29.96±2.18 (27.91-34.02)
Stylet knob height	2.78±0.43 (2.14-3.42)	2.66±0.36 (2.00-3.07)	3.23±0.56 (2.45-4.37)	2.94±0.31 (2.38-3.45)	3.02±0.35 (2.37-3.6)	2.77±0.46 (2.16-3.23)
Stylet knob width	5.82±0.48 (5.16-6.6)	5.92±0.5 (5.17-6.72)	5.67±0.73 (4.66-6.84)	5.87±0.54 (4.81-6.51)	5.71±0.51 (4.92-6.6)	6.61±0.73 (6.00-7.83)
Body diam. at stylet knob	19.1±0.89 (17.77-20.56)	17.93±0.6 (17.13-18.93)	18.58±0.86 (17.36-19.81)	20.13±1.64 (17.84-22.78)	19.13±0.88 (18.04-20.54)	20.24±1.49 (18.11-21.89)
Body diam. at middle bulb	25.52±1.93 (21.44-27.3)	22.36±1.33 (20.82-24.28)	25.37±1.49 (21.87-27.32)	26.85±2.24 (23.76-31.29)	25.6±1.95 (22.58-28.35)	25.16±2.33 (23.25-29.13)

Where: N = the numbers of individual nematodes were measured, L = body length (in μm), a = body length/maximum body width, b = body length/distance from anterior end to pharyngo-intestinal junction, c= body length/tail length, c’= tail length/body width at anus (female) or cloaca (male), V= Distance of vulva from anterior end x 100/total body length (%)

Tylenchorhynchus and *Merlinius* are the same clade and arrange in the Tylenchoidea group in both ITS1 and 18S regions (Figs. 5 and 6).

Discussion

This is a first record of *Tylenchorhynchus* and *Merlinius* associated with indigenous plants in China. *T. annulatus* and *T. thermophilus* were collected from Zijinggang and Tianmushan areas of Hangzhou, respectively. The climate conditions in these areas conform to the ecological characters of these nematodes. In fact, both are thermophilic and prefer 40 - 60% as optimum soil moisture, but can survive in the freezing conditions (Golden *et al.*, 1995). Molecular phylogenetic inference of ITS1 and 18S sequences of *T. annulatus* showed that the Chinese population is similar to 99.3% *T. annulatus* from Taiwan (Genebank accession numbers: EF030983) and 96.4% *T. maximus* from Belgium (Genebank accession numbers: AY993979) (Figs. 5 and 6). Meanwhile, molecular phylogenetic inference of ITS1 and 18S sequences of *T. thermophilus* showed that Chinese

Table 3: Nematodes in this study and genebank acession numbers

Species	Host	Location	Genebank accesion numbers (*)		Sources
			18S	ITS1	
Tylenchorhynchus annulatus	*Broussonetia papyrifera*	Hangzhou, China	TJG198	TJ1053	This study
T. thermophilus	*Pinus hwangshanensis*	Hangzhou, China	TBG115	TBG58	This study
Merlinus brevidens	*Bambusa multiplex*	Hangzhou, China	RS1198	-	This study
M. brevidens	*Stellera chamaejasme*	Shanxi, China	ST6129	ST1258	This study
M. brevidens	*Stellera chamaejasme*	Shanxi, China	ST1298	STB1217	This study
M. brevidens	*Ranunculus japonicas*	Shanxi, China	ST3112	MS2758	This study
M. brevidens	*Radix ex Rhizoma veratri*	Shanxi, China	ST4981	-	This study
M. brevidens	*Aconitum tongolense*	Shanxi, China	MTS129	STM558	This study
Tripylina sp.	*Broussonetia papyrifera*	Hangzhou, China	KC 109737 (*)	FJ 1258	This study
Tripylina sp.	*Broussonetia papyrifera*	Hangzhou, China	KC130182(*)	TZ 1258	This study

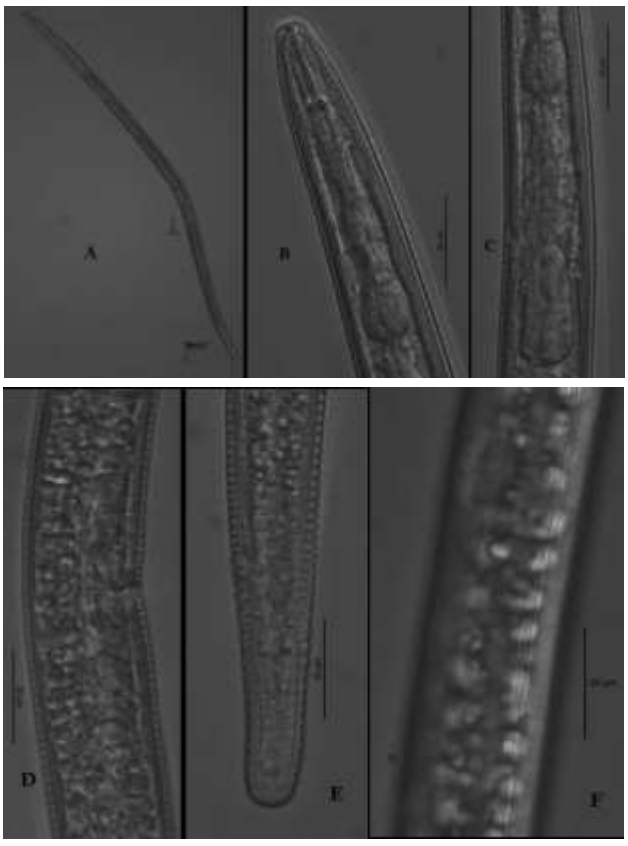

Fig. 1: Female of *Tylenchorhynchus thermophilus* Golden, Baldwin and Mundo- Ocampo, 1995 from Hangzhou, China; A: entire body, B: head, C: esophagus region, D: reproductive region, E: Tail region, F: lateral field

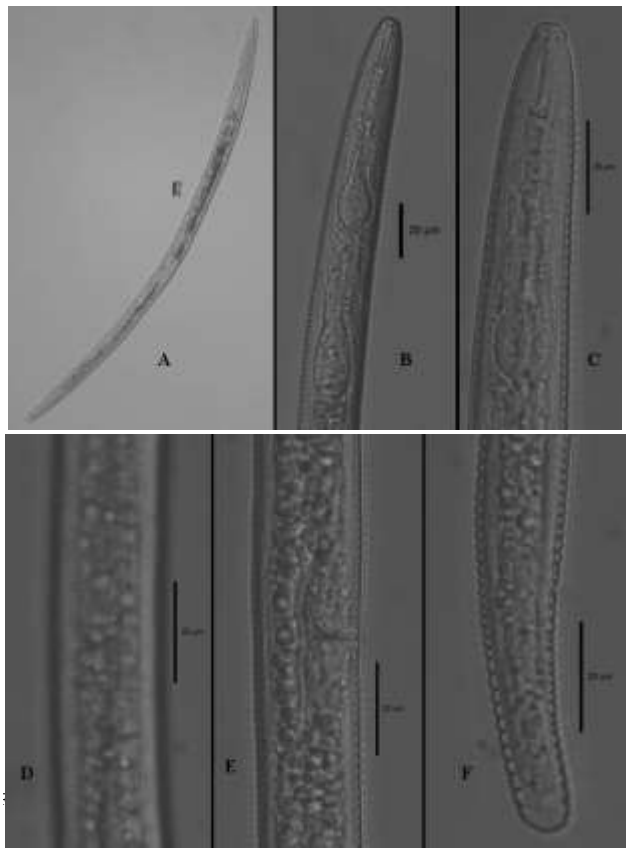

Fig. 2: Female of *Tylenchorhynchus annulatus* Golden, 1971 from Tianmushan, China; A: entire body, B, C: heads, D: lateral field, E: reproductive region, F: tail region

population is similar to 91.1% *T. annulatus* from Taiwan (Genebank accession numbers: EF030983) and 93.1% *T. maximus* from Belgium (Genebank accession numbers: AY993979) (Figs. 5 and 6).

The stylets of *M. brevidens* from Chinese populations are much longer than the previous descriptions (26 -34 µm vs 15 -16 µm) and actually, are as long as those of *M. graveti* from Bulgaria (Budurova, 1988), one of the longest stylets in the *Merlinius* genus. The difference of stylet length is possibly caused by the different geographical locations and hosts. Interestingly, all six populations of *Merlinius* from Chinese populations are identical 97. 4- 99.3% to *M. brevidens* (Genebank accession numbers: AY284597) and 97 - 99.2% to *M. joctus* (Genebank accession numbers: FJ969128) from the Netherlands. None of the sequence of *Merlinius* species on using ITS1 is released on Genebank data.

In conclusion, these species are not considered as economically or ecologically important to agricultural or forest plants as *Bursaphelunchus* spp., *Meloidogyne* spp., *Longidorus* spp. and *Pratylenchus* spp. However, studies to identify plant pests and pathogens are necessary to give

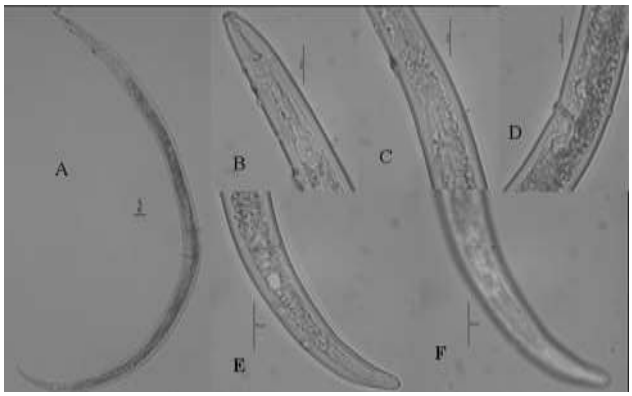

Fig. 3: Female of *Merlinius brevidens* Siddiqui, 1970 from China; A: entire body, B: head, C: esophagus region, D: reproductive region, E,F: Tails

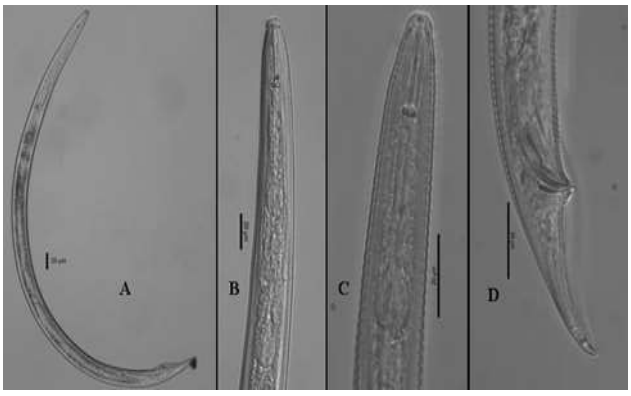

Fig. 4: Male of *Merlinius brevidens* Siddiqui, 1970 from China; A: entire body, B,C heads, D: tail

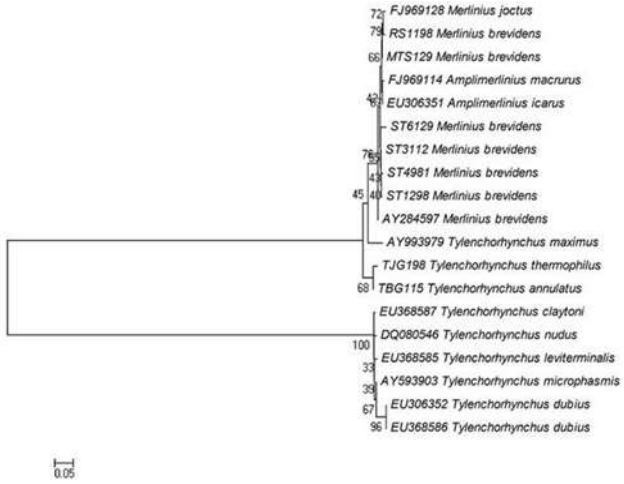

Fig. 5: Phylogenetic analysis of 18S region of *Tylenchorhynchus* and *Merlinius* from China and related species available in Genebank

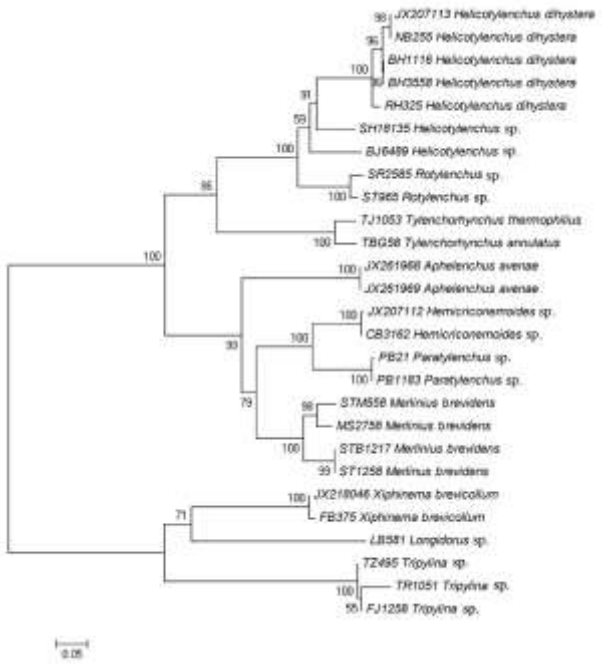

Fig. 6: Phylogenetic analysis of ITS1 region of the *Tylenchorhynchoidae* and other soil nematodes from China

proper strategies to control disease impacts. The results of this study not only provided the morphological and molecular characterizations of the *Tylenchorhynchus* and *Merlinius* species from China, but also demonstrate the increased geographical and host range of these nematodes.

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